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Targeting of Zyxin to Sites of Actin Membrane Interaction and to the Nucleus*

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The localization of proteins to particular intracellular compartments often regulates their functions. Zyxin is a LIM protein found prominently at sites of cell adhesion, faintly in leading lamellipodia, and transiently in cell nuclei. Here we have performed a domain analysis to identify regions in zyxin that are responsible for targeting it to different subcellular locations. The N-terminal proline-rich region of zyxin, which harbors binding sites for α-actinin and members of the Ena/VASP family, concentrates in lamellipodial extensions and weakly in focal adhesions. The LIM region of zyxin displays robust targeting to focal adhesions. When overexpressed in cells, the LIM region of zyxin causes displacement of endogenous zyxin from focal adhesions. Upon mislocalization of full-length zyxin, at least one member of the Ena/VASP family is also displaced, and the organization of the actin cytoskeleton is perturbed. Zyxin also has the capacity to shuttle between the nucleus and focal adhesion sites. When nuclear export is inhibited, zyxin accumulates in cell nuclei. The nuclear accumulation of zyxin occurs asynchronously with approximately half of the cells exhibiting nuclear localization of zyxin within 2.3 h of initiating leptomycin B treatment. Our results provide insight into the functions of different zyxin domains.

Cells change shape, migrate, proliferate, and differentiate in response to extracellular cues. Such sensitivity to environmental conditions is necessary for multiple processes including development, cell-mediated immunity, and organ regeneration (1, 2). Although much has been learned about a cell's capacity to recognize and respond to extracellular cues, in many cases we do not understand how engagement of a particular cell surface receptor triggers changes in cell behavior. One present challenge, for example, is to understand how engagement of integrin receptors for extracellular matrix can stimulate cell motility and changes in gene expression. In the past few years, several proteins that may contribute to these processes have been identified. For example, zyxin, a protein that is co-localized with integrins at sites of membrane-substratum adhesion, has properties that suggest it could participate in both localized actin polymerization and communication to the nuclear compartment.

Zyxin is an elongate phosphoprotein composed of three C-terminal LIM domains, a proline-rich N-terminal region, and at least one nuclear export signal (NES)[1] (Fig. 1*A*) (3–6). Two binding partners for the LIM region of zyxin, H-warts/LATS1 and members of the cysteine-rich protein (CRP) family, have been identified (5, 7). H-warts/LATS1 is a tumor suppressor that is postulated to be an important regulator of mitotic progression (7). CRPs are thought to stabilize actin-rich structural elements in muscle (8, 9). The N-terminal proline-rich region of zyxin displays docking sequences for several proteins implicated in actin assembly and organization including α-actinin and members of the Ena (enabled)/VASP (vasodilator-stimulated phosphoprotein) family (10–16). α-Actinin is a cross-linker of filamentous actin and a dynamic constituent of focal adhesions (17). Ena/VASP family members are profilin-binding proteins that have been proposed to regulate actin assembly and dynamics (13, 18–21).

Although the molecular details remain to be elucidated, zyxin's binding partner repertoire implicates it in actin assembly and organization. Results from several lines of investigation are compatible with this possibility. For example, targeting of zyxin to the plasma membrane results in the elaboration of actin-rich cell surface projections (22), and actin polymerization is induced in association with mitochondria when zyxin is targeted to the organelle surface.[2] In a more global sense, zyxin appears to participate in cell movements that are known to depend on actin. Antibody-mediated inhibition of zyxin function results in disturbances in cell spreading, cell locomotion, and filopodial maintenance.[3] Similarly, a peptide inhibitor of zyxin function, which causes it to be mislocalized in cells, also disturbs cell spreading and migration (12).

In addition to its cytoskeletal role, zyxin has also been postulated to participate in intracellular communication between

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[1] The abbreviations used are: NES, nuclear export signal; CRP, cysteine-rich protein; PCR, polymerase chain reaction; aa, amino acids; GFP, green fluorescent protein; GST, glutathione *S*-transferase.

[2] J. Fradelizi, V. Noireaux, B. Menichi, D. Louvard, J. Prost, C. Sykes, R. M. Golsteyn, and E. Friederich, submitted for publication.

[3] B. A. Benson, F. Wang, D. G. Jay, and M. C. Beckerle, submitted for publication.

the plasma membrane and the nucleus (4). Avian zyxin displays a leucine-rich nuclear export signal and can shuttle between the nucleus and sites of cell-substratum adhesion (4). Two other zyxin family members, the lipoma-preferred partner, which is present in a mutant form in many lipomas (25, 26), and the thyroid receptor-interacting protein, Trip-6, are also constituents of focal adhesions that display nuclear export signals (27–29). Although the precise role of zyxin family members within cell nuclei is not understood, both Trip6 and lipoma-preferred partner have the capacity to activate transcription under some experimental conditions (27, 29); thus, zyxin family members may directly influence gene expression.

Because zyxin appears to have distinct functions in association with the actin cytoskeleton and in the cell nucleus, the appropriate localization of zyxin to discrete subcellular domains is likely to be a critical determinant of its function. To better understand how the subcellular localization of zyxin is specified, we introduced a series of zyxin truncation proteins into cells and monitored their intracellular distributions. By this approach, we have identified several regions of the zyxin protein as key determinants of its subcellular localization.

EXPERIMENTAL PROCEDURES

Eukaryotic Expression Plasmid Construction—Plasmids used to express avian zyxin proteins in eukaryotic cells were created by subcloning polymerase chain reaction (PCR)-amplified chicken zyxin cDNA (5) into the pcDNA1/Neo eukaryotic expression vector from Invitrogen (San Diego, CA) (30). Site-directed mutagenesis was performed with Promega's Altered Sites kit (Madison, WI) to create PCR template for the deletion mutant cZyxΔ322–331. PCR stitching was used to create the cZyxΔ24–46 insert. This entails initially amplifying two fragments of zyxin that lack sequences encoding aa 24–46 but contain regions of homology in their primers that enable annealing and extension in a second round of PCR amplification.

FLAG-tagged constructs were created by cloning PCR-derived template into a modified pcDNA1/Neo construct containing sequences designed to express zyxin protein fused with a C-terminal FLAG epitope tag (DYKDDDDK). The FLAG epitope allowed us to visualize the localization of zyxin deletion variants that could not be detected by existing anti-zyxin antibodies. The addition of a C-terminal FLAG epitope sequence had no effect on the ability of zyxin or its deletion variants to localize properly in cells.

For GFP-tagged zyxin, DNA encoding all or part of zyxin was inserted into the pEGFP-C1 vector (CLONTECH Laboratories), and the DNA encoding the GFP-fusion protein was transferred into the pUHD 10-3 vector (31) for improved expression. GFP-hZyx (F71A,F93A, F104A,F114A) was constructed in four steps by site directed mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene).

RCAS proviral plasmids engineered to express FLAG epitope-tagged chicken zyxin protein in chicken cells were created by cloning PCR-derived zyxin-FLAG sequences into the *Cla*I restriction endonuclease site of the RCAS A (BH) vector as described previously (32).

All constructs were sequence-verified prior to use.

Glutathione S-Transferase (GST) Fusion Protein Expression—GST-fusion protein expression constructs were created by cloning PCR-derived chicken zyxin sequences into pGEX2T-128/129 as previously described (33). GST-zyxin fusion proteins were produced by inducing log phase BL21(DE3) bacteria containing the pGEX2T-128/129 construct with isopropyl-1-thio-β-D-galactopyranoside for 2 h, purifying the recombinant protein with glutathione-agarose beads, and concentrating the eluate with a Centricon-10 concentrator (Amicon, Beverly, MA). The concentrate was equilibrated in calcium-free phosphate-buffered saline prior to microinjection.

Transfection, Microinjection, and Infection—The human cervix carcinoma HeLa cell line (ATCC CCL-2) and the African green monkey kidney Vero cell line (ATCC CCL-81) were grown in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 2 mM glutamine and 10% fetal calf serum at 37 °C and 10% CO_2. HeLa and Vero cells were transfected using the calcium phosphate DNA precipitation method. 2 μg of pUHD 10-3 vector in which expression of the cDNA is under the control of a minimal promoter were co-transfected with 2 μg of pUHD 15-1 plasmid, which encodes the tTAs transactivator. Cells were analyzed 24 h after the addition of DNA. The rat embryo fibroblast cell line, REF-52 (34) was grown on glass coverslips to ~80% confluency prior to microinjection using an Eppendorf Micromanipulator/Transjector apparatus (Madison, WI). Cesium chloride-purified plasmids were microinjected at a concentration of 0.2–0.25 mg/ml in calcium-free phosphate-buffered saline. Recombinant GST fusion proteins were mixed with fluorescein isothiocyanate-labeled bovine serum albumin (Molecular Probes, Inc., Eugene, OR) and injected at a final concentration of 5–10 mg/ml in calcium-free phosphate-buffered saline for each protein. Primary chicken embryo fibroblasts were isolated and cultured as described (35). Proviral RCAS expression vectors were introduced into chicken embryo fibroblasts by calcium phosphate transfection and cultured for 5 days to allow for complete infection prior to visualizing zyxin protein expression.

Immunofluorescence—Indirect immunofluorescence was performed using established procedures. Antibodies utilized in this study included the following: a rabbit polyclonal anti-zyxin antibody B38 (1:400) (6); a chicken-specific anti-zyxin mouse monoclonal antibody, m1334 (undiluted tissue culture supernatant) (24); an anti-β-galactosidase monoclonal antibody GAL-13 (1:4000) (Sigma); the anti-FLAG epitope mouse monoclonal antibody M2 (1:2000) (IBI/Kodak/VWR Scientific, Inc., New Haven, CT); an anti-FLAG epitope affinity-purified rabbit polyclonal antibody (1:40); an anti-Myc epitope monoclonal antibody 1–9E10.2 (1:1500 dilution of ascites fluid) (ATCC, Manassas, VA); an anti-vinculin monoclonal antibody hVin-1 (1:400) (Sigma); anti-α-actinin monoclonal antibodies BM75.2 (1:50) (ICN, Costa Mesa, CA) and 637941 (1:50) (ICN); an anti-Mena rabbit polyclonal antibody 2197(1:400) (Frank Gertler); an anti-VASP monoclonal antibody C43 (1:100) (Transduction Laboratories) and several fluorochrome-labeled secondary antibodies; Texas Red goat anti-rabbit Ig (1:200); fluorescein isothiocyanate goat anti-mouse Ig (1:500) (Cappel, Durham, NC); Texas Red goat anti-mouse IgG (1:100); and Texas Red goat anti-mouse IgG + IgM (H + L) (1:50) (Jackson ImmunoResearch Laboratories). Fluorochrome-labeled phalloidin (1:400) (Molecular Probes) was used to detect polymerized actin. The B38 anti-peptide antiserum used to stain for endogenous zyxin recognizes both zyxin and the zyxin family member lipoma-preferred partner (data not shown; Ref. 6). Zyxin expression is roughly 10-fold greater than that of lipoma-preferred partner in fibroblast cells (36).

Cells were photographed with a Zeiss Axiophot Microscope (New York) or captured using a PI CCD camera (Princeton Instruments, Trenton, NJ). Negatives were digitized, and images were processed using Adobe Photoshop (Mountain View, CA) software.

Nuclear Export Inhibition—HeLa cells transiently transfected for GFP-hZyx were treated with 20 nM leptomycin B (B. Wolff-Winiski, Novartis, Basel, Switzerland) and fixed 0, 90, 180, or 360 min after the addition of the drug.

RESULTS

Zyxin displays an N-terminal proline-rich region and three C-terminal LIM domains that provide binding sites for several proteins associated with actin polymerization and intracellular signaling (Fig. 1*A*). The binding sites for both α-actinin and Ena/VASP proteins have been precisely mapped within the proline-rich region (12, 14, 16, 37). CRP1 and the H-warts/LATS1 tumor suppressor bind to elements in the LIM region (7, 33). Zyxin is found in focal adhesions and leading lamellipodia (11, 15),[4] but it can also travel to the cell nucleus and exhibits one or more nuclear export signals (4).[5] We used a mutagenesis strategy to define the critical domains in zyxin necessary for targeting the protein to these subcellular compartments.

Identification of Zyxin Sequences Involved in Focal Adhesion Targeting—We focused first on an effort to map the domain(s) of zyxin required for targeting of the protein to focal adhesions. We have compared the subcellular distributions of full-length human and chicken zyxin to the distributions of several deletion variants and site-directed mutants (Fig. 1, *B* and *C*). GFP-tagged human zyxin displays a distribution pattern that is indistinguishable from that of the endogenous protein. At steady state, no protein is detected within cell nuclei. Rather,

[4] K. Rottner, M. Krause, M. Geese, M. Gimona, A. Sechi, J. V. Small, and J. Wehland, submitted for publication.

[5] D. A. Nix, J. Fradelizi, S. Bockholt, B. Menichi, D. Louvard, E. Friederich, and M. C. Beckerle, unpublished observations.

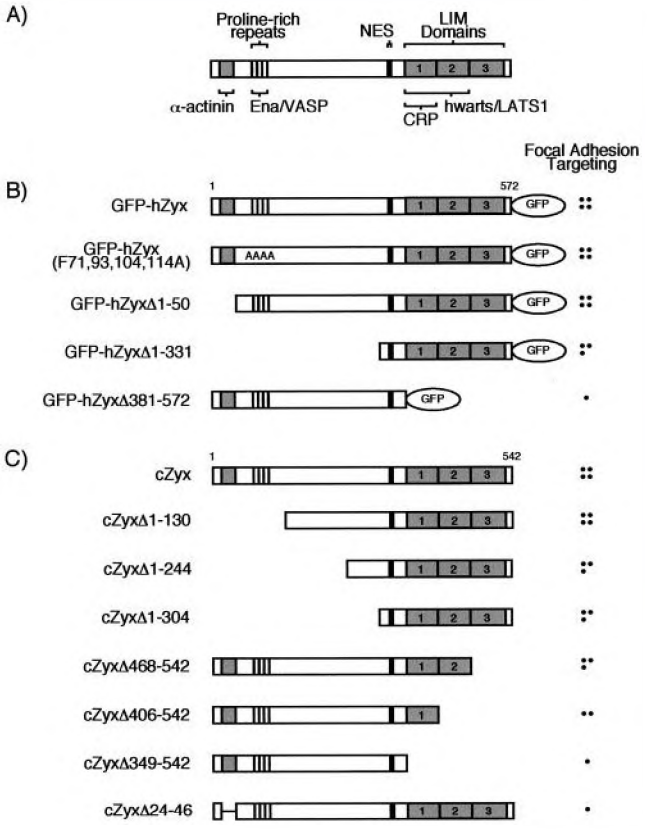

FIG. 1. **Zyxin structure and focal adhesion targeting.** *A*, zyxin's primary structure and published binding partners. *B*, GFP-tagged human zyxin; *C*, chicken zyxin deletion constructs used in this study. The incorporation of an epitope tag at the C terminus of two zyxin constructs, cZyxΔ1–244 and cZyxΔ1–304, yielded indistinguishable results. GFP-hZyx constructs were introduced into Vero cells by transient transfection. cZyx proteins were introduced into REF-52 cells by microinjecting zyxin expression constructs. The localization pattern of the expressed zyxin variants was determined by scoring transfected cells for focal adhesion staining. *Four dots* represent strong localization of the expressed protein comparable with what is observed for endogenous protein. A *decreasing number* of *dots* indicates progressively less localization of the expressed construct. A *single dot* represents very poor to no ability to localize at focal adhesions.

the protein is associated with the focal adhesions, the leading edge, and periodically along the actin stress fibers (Fig. 2, *A* and *B*). Within the leading edge, zyxin is present in small punctate patches against a diffuse background of accumulated protein (Fig. 2*A*, *arrow*). Confocal microscopy reveals that there is a *bona fide* concentration of zyxin within the actin-rich zone of lamellipodial projections above the level found in cytoplasm (data not shown). As observed previously in immunocytochemical studies, GFP-zyxin co-localizes with VASP and α-actinin in focal adhesions and is often observed in a periodic distribution along stress fibers (11, 16, 35). This periodic localization of zyxin on stress fibers is particularly striking in cell types that have well developed bundles of actin filaments, such as the Vero cells shown in Fig. 2. In addition, the ability to detect zyxin in association with stress fibers appears enhanced when visualizing live cells that express GFP-tagged zyxin, as compared with fixed specimens prepared for indirect immunofluorescence. This may reflect some difficulty with antibody accessibility in fixed material.

To identify zyxin sequences that target it to focal adhesions, we first examined the effect of perturbing zyxin's ability to bind Ena/VASP proteins or α-actinin. Zyxin interacts with Ena/VASP proteins via four proline-rich repeats with the consensus (D/E)FPPPP (Fig. 1*A*; Refs. 14 and 37)). Previous work has demonstrated that substitution of the conserved Phe residue with Ala completely eliminates Ena/VASP binding capacity both *in vitro* and *in vivo* (14, 37). Compromising all four of zyxin's Ena/VASP binding sites resulted in no detectable inhibition of its ability to target to focal adhesions (Fig. 2, *C* and *D*). Likewise, deletion of residues 1–50, which contain the α-actinin binding site (12, 16), caused no appreciable inhibition of focal adhesion targeting (Fig. 2, *E* and *F*). Furthermore, deletion of residues 1–331, which eliminates nearly all sequence information outside the LIM region, did not eliminate focal adhesion targeting (Fig. 2, *G* and *H*), although we typically observed a higher level of diffuse cytoplasmic localization than with full-length protein. In contrast, elimination of the LIM region caused a dramatic reduction in focal adhesion targeting capacity (Fig. 2, *I* and *J*).

These results suggested that none of zyxin's binding partners that dock on the proline-rich N-terminal domain are absolutely required for the targeting of human zyxin to focal adhesions. Our findings contrasted with those in a recent report (16) in which precise deletion of zyxin's α-actinin binding site was observed to impair focal adhesion targeting. We therefore sought to clarify the role of α-actinin binding for subcellular targeting of zyxin by defining sequences required for focal adhesion localization of zyxin in a second species. Consistent with what we observed for human zyxin (Fig. 2), deletion constructs that eliminated up to 304 residues from the N terminus of chicken zyxin retained the capacity to target to focal adhesions (Fig. 1*C* and Fig. 3, *C–H*). Although subtle reductions in the efficiency of zyxin targeting to focal adhesions are impossible to quantify using this approach, examination of a large number of cells supports the conclusion that the N-terminal domain is not essential for the focal adhesion targeting. As noted above, this conclusion contrasts with that of Reinhard *et al.* (16), who concluded that zyxin's α-actinin binding site represents an essential focal adhesion targeting determinant. In that study, an internal deletion of amino acid residues corresponding to the α-actinin binding site was made, and it was noted that focal adhesion targeting of zyxin was impaired. In our case, larger N-terminal deletions failed to eliminate focal adhesion targeting. In order to compare directly our system with that of Reinhard *et al.*, we generated the comparable internal deletion construct in which amino acid residues 24–46 were eliminated from chicken zyxin. This region of chicken zyxin is 83% identical and 96% similar to the comparable sequence in human zyxin that was deleted by Reinhard *et al.* (16) (Fig. 3*M*). Consistent with the report by Reinhard *et al.*, we also observed a failure of zyxin lacking this sequence to target effectively to focal adhesions (Fig. 3, *K* and *L*), although robust expression was observed (data not shown). Because more extensive deletions of N-terminal sequences fail to compromise significantly the ability of zyxin to target to focal adhesions, targeting to these regions must involve a more complex mechanism than simple docking of zyxin at α-actinin-rich sites.

Interestingly, although deletion of the N-terminal two-thirds of zyxin failed to eliminate focal adhesion targeting, sequential deletion of individual LIM domains did result in a progressive loss of focal adhesion targeting capacity (Fig. 1*C* and data not shown). Strikingly, a construct lacking all three LIM domains showed only a rudimentary capacity to localize to focal adhesions (Fig. 3, *I* and *J*). These data illustrate that the LIM region of zyxin contains the primary focal adhesion targeting elements. Site-directed mutagenesis of the tyrosine residues within zyxin's LIM region failed to affect its ability to localize at focal adhesions (data not shown). Thus, although tyrosine kinases and their substrates are enriched at cell-substratum adhesion sites (1), tyrosine phosphorylation within the LIM

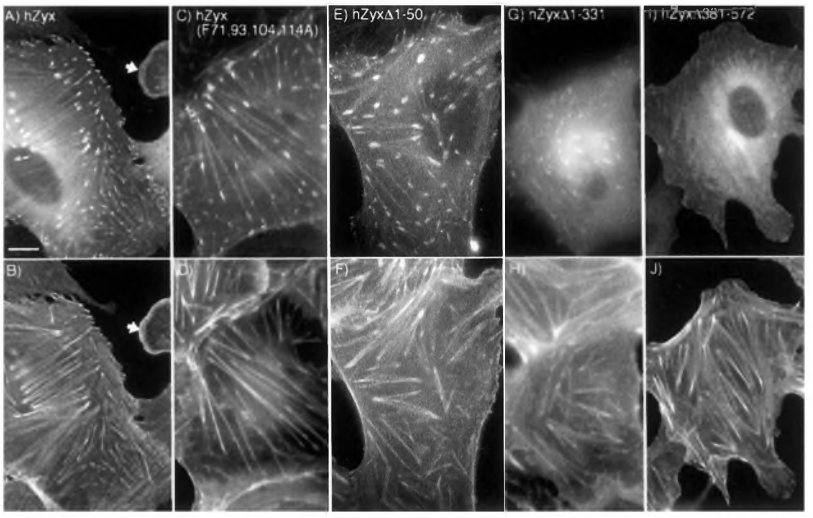

FIG. 2. **Focal adhesion targeting domains of human zyxin.** Vero cells transiently transfected with GFP-hZyx (*A* and *B*), GFP-hZyx (F71A,F93A,F104A,F114A) (*C* and *D*), GFP-hZyxΔ1–50 (*E* and *F*), GFP-hZyxΔ1–331 (*G* and *H*), or GFP-hZyxΔ381–572 (*I* and *J*) were fixed and labeled for F-actin by rhodamine-phalloidin. Cells were visualized either for GFP (*A*, *C*, *E*, *G*, and *I*) or phalloidin (*B*, *D*, *F*, *H*, and *J*) labeling. *Bar*, 10 μm.

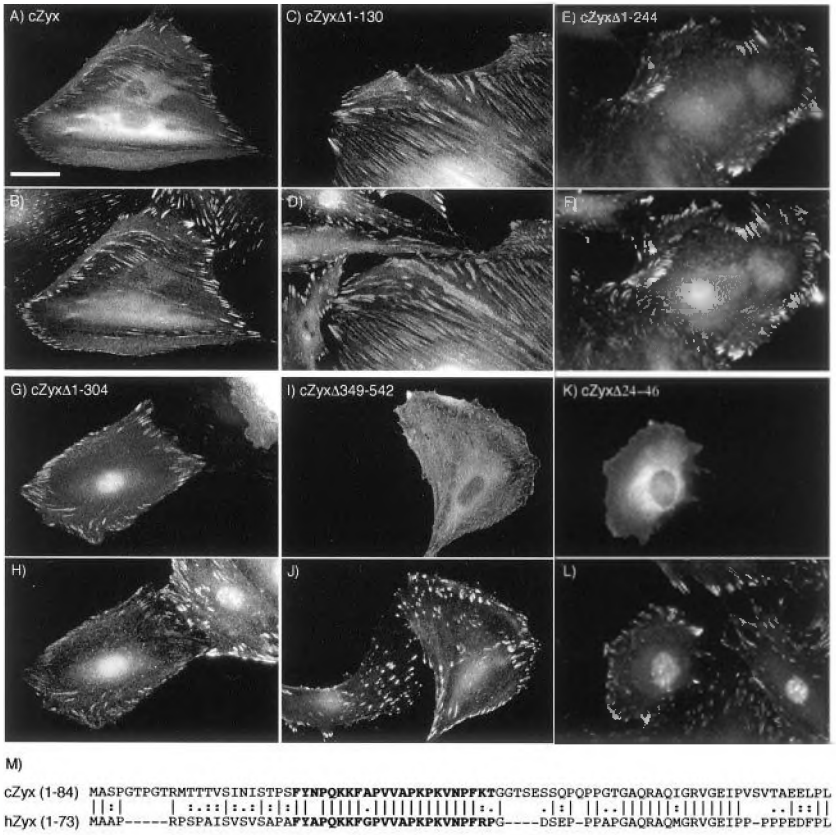

FIG. 3. **Focal adhesion targeting domains of chicken zyxin.** REF-52 cells microinjected with cZyx (*A* and *B*), cZyxΔ1–130 (*C* and *D*), cZyxΔ1–244 (*E* and *F*), cZyx Δ1–304 (*G* and *H*), cZyxΔ349–542 (*I* and *J*), or cZyxΔ24–46 (*K* and *L*) were fixed and stained for either expressed zyxin with the chicken zyxin-specific antibody m1334 (*A*, *C*, *I*, and *K*) or an anti-FLAG antibody (*E* and *G*) and for endogenous zyxin (*B*, *D*, *F*, *H*, *J*, and *L*). In *E* and *G*, one can see that focal adhesion targeting is not highly efficient in every injected cell; quantitative analysis of the focal adhesion targeting efficiencies of the various constructs is reflected in the targeting scores provided in Fig. 1. A ClustalW alignment of the N-terminal portion of chicken and human zyxin is presented in *M* with the consensus α-actinin binding site highlighted in *boldface type*. *Bar*, 50 μm.

region of zyxin does not appear to regulate its targeting capacity.

In an effort to define which LIM domains target zyxin to focal adhesions, we injected a series of GST-LIM domain fusion proteins into cells (Fig. 4*A*; Ref. 33). Consistent with what we had observed for GFP-tagged and epitope-tagged zyxin variants, we detected robust incorporation of microinjected GST-LIM1–3 into the focal adhesions (Fig. 4*B*). Constructs displaying two LIM domains, either GST-LIM1–2 (Fig. 4*C*) or GST-LIM2–3 (Fig. 4*D*), were also detected in focal adhesions, albeit at lower levels than for GST-LIM1–3. None of the fusion proteins harboring an individual zyxin LIM domain accumulated in the focal adhesions (Fig. 4, *E–G*). Thus, the isolated LIM region of zyxin is sufficient to target zyxin to focal adhesions, and individual LIM domains appear to cooperate to allow maximal accumulation of zyxin at these sites.

Sequences in the Proline-rich N Terminus of Zyxin Recognize Docking Sites in the Leading Edge—During the course of the domain mapping studies in which we used transient transfection to visualize either GFP- or epitope-tagged zyxin, we occasionally noted that elimination of the LIM domains seemed to stimulate accumulation of the residual zyxin sequences at the leading edge of spreading/migrating cells, suggesting that some leading edge localization determinant was present in the N-terminal domain. This possibility was difficult to evaluate in transiently transfected cells that were well spread, because substantial lamellipodial extensions are not common in such cells. Therefore, we established an avian retroviral expression system that allowed us to look at large numbers of cells in the process of spreading, where lamellipodial projections are prominent (Fig. 5). As expected, the retrovirally programmed expression of full-length zyxin resulted in the localization of zyxin

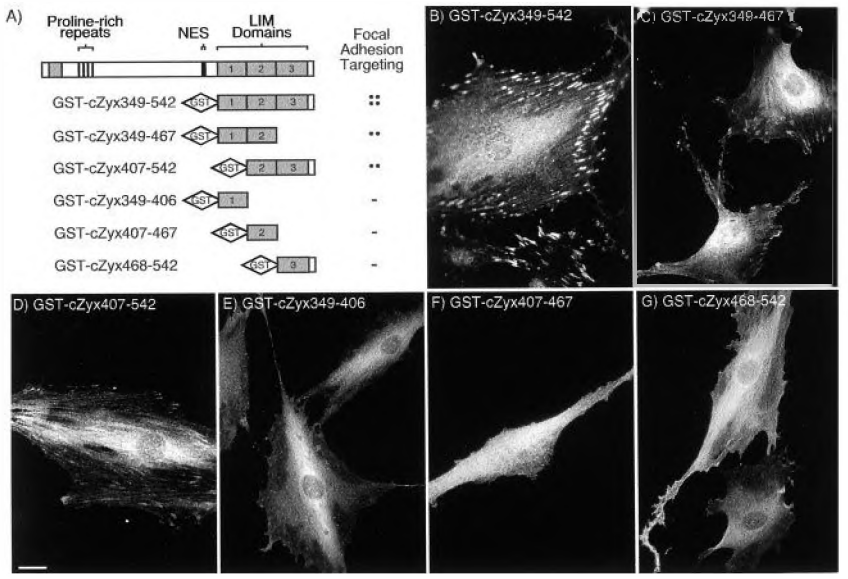

FIG. 4. **Multiple LIM domains of cZyx cooperate to target zyxin to focal adhesions.** A summary of GST-cLIM fusions used and their focal adhesion targeting capacity is shown in *A*. Fusion proteins GST-cZyx349–542 (*B*), GST-cZyx349–467 (*C*), GST-cZyx407–542 (*D*), GST-cZyx349–406 (*E*), GST-cZyx407–467 (*F*), or GST-cZyx468–542 (*G*) were purified from bacteria, microinjected into REF-52 cells, and visualized by immunofluorescence after fixing and staining for the FLAG epitope in the GST leader peptide. *Bar*, 20 μm.

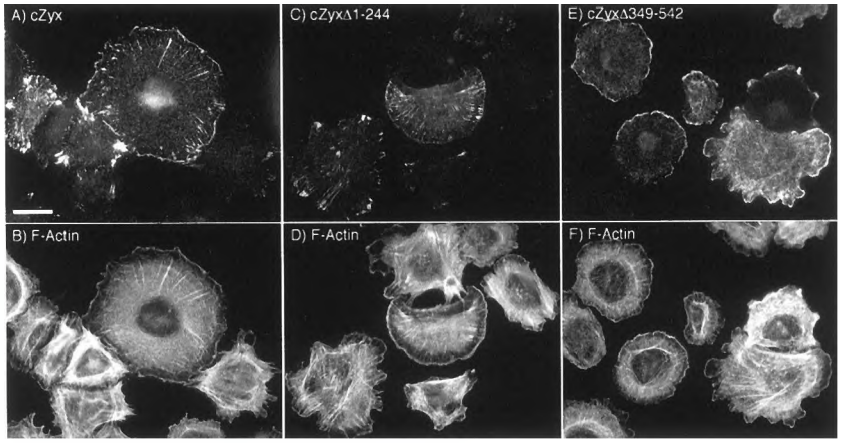

FIG. 5. **Zyxin's proline-rich region can accumulate in leading lamellae.** Primary cultured chicken embryo fibroblast cells were transfected with a proviral RCAS construct encoding full-length cZyx (*A* and *B*), cZyxΔ1–244 (*C* and *D*), or cZyxΔ349–542 (*E* and *F*). The cells were trypsinized and allowed to spread on fibronectin-coated coverslips for 30 min. Fixed cells were stained for exogenous zyxin with an anti-FLAG epitope antibody (*A*, *C*, and *E*) and for polymerized actin by phalloidin (*B*, *D*, and *F*). *Bar*, 20 μm.

to both the focal adhesions and the lamellipodia (Fig. 5, *A* and *B*). In contrast, an N-terminal zyxin truncation (ZyxΔ1–244) exhibited prominent focal adhesion concentration and was essentially absent from the lamellipodial extensions, although numerous actin rich lamellipodial protrusions were evident (Fig. 5, *C* and *D*). A complementary result was obtained for zyxin lacking the LIM region (ZyxΔ349–542); in this case, the lamellipodial extensions were particularly rich in zyxin, and focal adhesion labeling was minimal (Fig. 5, *E* and *F*).

Dominant Negative Interference with the Targeting of Zyxin to Focal Adhesions Affects Actin Organization and Cell Morphology—Because the LIM region of zyxin has the capacity to target to focal adhesions with high efficiency, we evaluated the possibility that it might be able to displace the endogenous zyxin from these sites. If that were the case, then it would be possible to populate focal adhesions with zyxin LIM sequences uncoupled from domains involved in docking α-actinin and Ena/VASP family members as well as other partners that bind the proline-rich N-terminal region of zyxin. By labeling transiently transfected cells with an anti-zyxin antibody, we showed that the presence of high levels of either GFP-tagged (Fig. 6, *A* and *B*) or epitope-tagged (Fig. 6, *C* and *D*) versions of zyxin's LIM region had the ability to displace the endogenous complement of zyxin from focal adhesions. In the case of cZyxΔ1–304 expression, endogenous zyxin was depleted from focal adhesions in 98% of the high expressing cells examined. Although zyxin is substantially mislocalized, vinculin retained a normal focal adhesion distribution in the majority of cells examined (Fig. 6, *E* and *F*). Similarly, α-actinin could still be observed in association with actin stress fibers (Fig. 6, *G* and *H*), although, as will be discussed below, the actin arrays were often altered. In contrast, Mena was significantly reduced in the focal adhesions in 90% of the high expressing cells examined when endogenous zyxin was displaced from those sites (Fig. 6, *I* and *J*). We also made a substantial effort to evaluate the consequences of overexpressing zyxin that lacks LIM domains (*i.e.* the N-terminal region). However, after microinjection of thousands of individual cells, we recovered only about 1% of the injected cells, suggesting that long term overexpression of this region of zyxin has adverse effects on cell viability. In contrast, overexpression of a control protein, β-galactosidase, had no detectable impact on cell viability or on the focal adhesion composition or the integrity of the actin cytoskeleton (Fig. 6, *K* and *L*, and data not shown), illustrating that the observed effects are specific to the presence of zyxin sequences and not simply caused by the expression of a foreign protein.

The displacement of full-length zyxin by the LIM region construct results in the elimination of zyxin-dependent docking sites for proteins, including Ena/VASP family members, that bind to the proline-rich N terminus of zyxin. Therefore, we were able to probe the importance of this region of zyxin by examining the effect of the zyxin-LIM construct on the integrity of the actin cytoskeleton and on general cell morphology. We observed that the cells that expressed the LIM region of zyxin often appeared less well spread than their counterparts (Fig. 6), and the majority were markedly retracted at later time

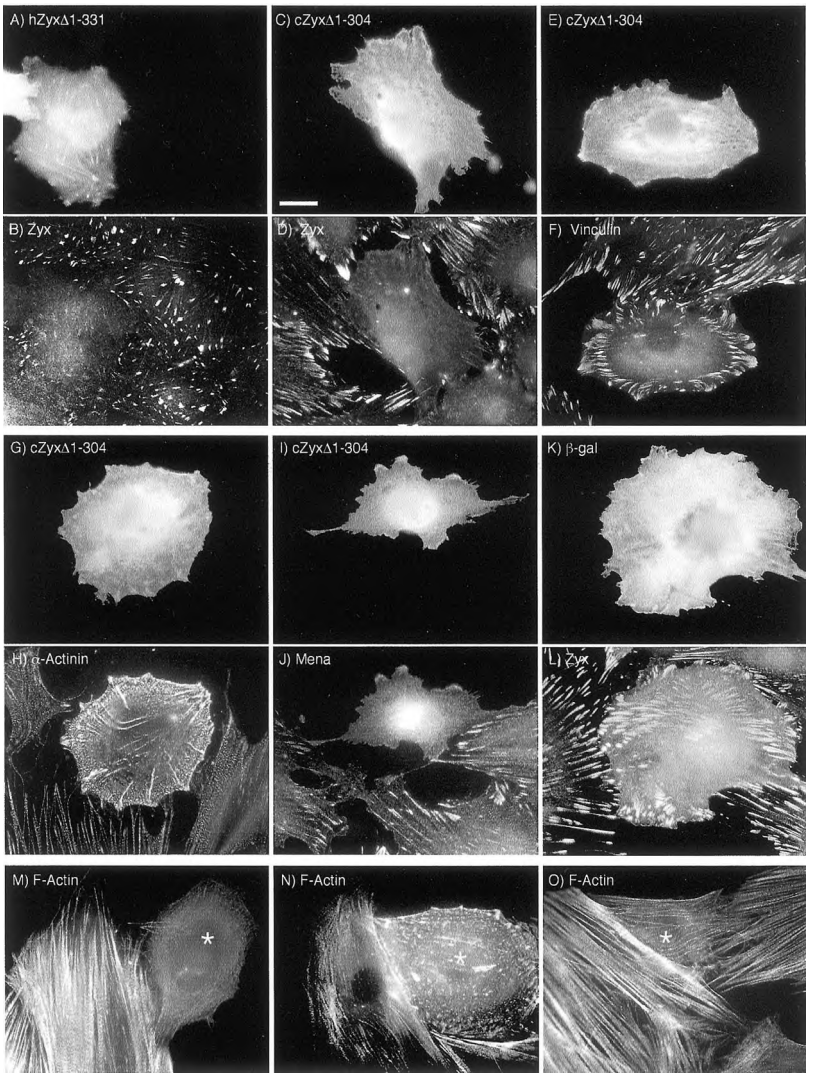

FIG. 6. **Overexpression of the LIM region of zyxin causes displacement of endogenous zyxin and disturbs focal adhesion composition and actin organization.** *A* and *B*, Vero cells transiently transfected with GFP-hZyxΔ1–331 were fixed and visualized either for GFP (*A*) or for endogenous zyxin (*B*). GFP-hZyxΔ1–331 accumulates at focal adhesions (*A*) with a corresponding loss of the endogenous full-length protein (*B*) from these sites. *C–O*, REF-52 cells programmed to express cZyxΔ1–304 (*C–J* and *M–O*) or β-galactosidase (*K* and *L*) were visualized by fluorescence microscopy to detect either the expressed FLAG-tagged protein (*C*, *E*, *G*, *I*, and *K*) or endogenous zyxin (*D* and *L*), vinculin (*F*), α-actinin (*H*), Mena (*J*) or F-actin (*M–O*). In *M–O*, *asterisks* indicate the injected cells. *Bar*, 50 μm.

points when expression levels were high (data not shown). In addition, the architecture of the actin cytoskeleton was disturbed in cells that expressed the dominant negative zyxin (Fig. 6, *M–O*). These results illustrate that the proline region of zyxin harbors determinants that play a critical role in the localization of Ena/VASP family members and probably other partners to focal adhesions. In the absence of this region of zyxin, actin stress fibers are fewer in number, thinner, and often fragmented, suggesting that the assembly and/or maintenance of actin bundles depends on properly localized full-length zyxin.

Multiple Sequences Can Target Zyxin to the Nucleus—In previous work, we showed that chicken zyxin displays a nuclear export signal (NES) and shuttles between the nuclear and cytoplasmic compartments of cells (4). Since no obvious nuclear localization signals are present in zyxin, it is likely that some protein partner or partners carry the protein into the nucleus. To learn more about the mechanism by which zyxin enters the nucleus, we evaluated the capacity of several zyxin deletion variants to accumulate in cell nuclei (Fig. 7*A*). Zyxin is not typically detected in cell nuclei at steady state (Fig. 7*B*); however, we do occasionally detect cells (7% of the total; 14 of 208 cells scored) in which expressed full-length zyxin is found in the nucleus. This observation suggests that cells may be modulating the subcellular distribution of zyxin in response to a physiological cue. Elimination of the core elements of the NES, residues 322–331 in chicken zyxin, reliably results in nuclear accumulation of the protein (Fig. 7*C*). Deletion of the NES from zyxin does not disturb the ability of cytoplasmically located protein to associate normally with the actin cytoskeleton. When the entire proline-rich region (aa 1–348), including the NES, is removed, nuclear accumulation of the expressed LIM domain triplet is observed in all cells (Fig. 7*D*). Exogenous expression of other LIM domains, which are also small enough to diffuse across the nuclear membrane, are not observed to concentrate in cell nuclei (39, 40); thus, the nuclear accumulation of the zyxin LIM region appears to be specific. To evaluate whether the proline-rich region of chicken zyxin might also contribute to localizing zyxin to the nucleus, we expressed the proline-rich region with or without its NES. The proline-rich region of zyxin is excluded from cell nuclei (Fig. 3*I*); however, if the NES is eliminated from the proline region, it too has the capacity to accumulate in cell nuclei (Fig. 7*E*). These data demonstrate that both the LIM and proline-rich regions of zyxin harbor sequences that can independently direct the protein to the nucleus.

Regulation of Zyxin's Accumulation within Cell Nuclei—In order to gain insight into the control of zyxin's access to the nuclear compartment, we examined the accumulation of GFP-zyxin within cell nuclei during treatment of cells with the nuclear export inhibitor, leptomycin B. As described earlier, GFP-zyxin is not typically detected in cell nuclei under normal culture conditions (Fig. 8*A*). However, upon the addition of leptomycin B, GFP-hZyx begins to accumulate within cell nu-

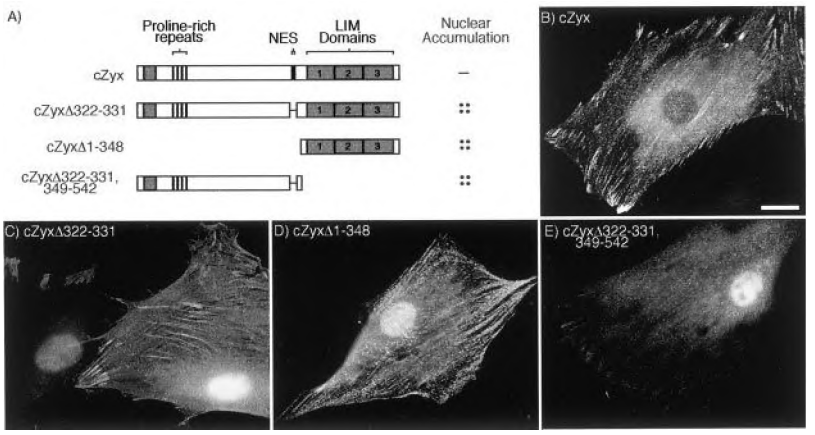

FIG. 7. **Zyxin harbors multiple regions that support nuclear accumulation.** *A*, summary of cZyx variants employed and their nuclear accumulation. REF-52 cells programmed to express cZyx (*B*), cZyxΔ322–331 (*C*), cZyxΔ1–348 (*D*), or cZyx Δ322–331, 349–542 (*E*) were fixed and visualized for expressed zyxin. In the absence of the NES, zyxin is found in cell nuclei. Both the LIM region and the proline-rich region of zyxin are observed to accumulate within cell nuclei. *Bar*, 50 μm.

clei with kinetics that varied from cell to cell. Within 90 min of initiating the treatment, more than 25% of the cells displayed some evidence of increased zyxin in their nuclei (Fig. 8, *B* and *C*). Approximately half of the cells displayed nuclear zyxin within 2 h of inhibiting nuclear export with leptomycin B, and all cells displayed evidence of nuclear accumulation of zyxin within 6 h of initiating leptomycin B treatment. The asynchronous nature of the nuclear accumulation of zyxin in different cells suggests that the import of zyxin into a cell nucleus is responsive to individual cell physiologies.

DISCUSSION

The precise localization of a protein to a particular intracellular compartment is often essential for its normal function (41–43). Zyxin resides in three discrete compartments within cells: focal adhesions, lamellipodia, and the nucleus (3, 4, 35). We have performed a deletion analysis in an effort to define the regions of zyxin that are important for localizing it to discrete subcellular domains.

Multiple Sites Contribute to Focal Adhesion Targeting of Zyxin—The LIM domain is a double zinc finger structure involved in protein-protein interaction (33, 44, 45). LIM domains have been postulated to control protein complex assembly and function; here we provide an example in which the LIM sequences are utilized to specify subcellular distribution of a protein. Fusion proteins containing multiple zyxin-derived LIM domains, either LIM1–3, LIM1–2, or LIM2–3 had the capacity to localize to focal adhesions. In contrast, individual LIM domains failed to target focal adhesions, although they have been shown to retain function when expressed *in vitro* (33). This result is unlike what was observed for another focal adhesion protein, paxillin, for which one of the protein's four LIM domains was found to harbor the primary focal adhesion localization information (40). In the case of zyxin, multiple LIM domains are necessary for stable association with focal adhesions, at least when the full-length endogenous protein remains present in the cell. Given the ability of individual LIM domains to function independently in protein-protein interactions, this suggests that multiple simultaneous interactions may be required to stabilize the localization of zyxin at focal adhesions.

To date, two proteins have been shown to dock on the LIM region of zyxin. CRP family members bind exclusively to zyxin's first LIM domain (9, 33). Our mapping studies revealed that this domain is not absolutely essential for the focal adhesion localization of zyxin. Moreover, CRPs are not completely colocalized with zyxin in fibroblasts (5, 9). Therefore, CRPs cannot be solely responsible for docking zyxin at focal adhesions. The H-warts/LATS1 tumor suppressor has also been shown to interact with the LIM region of zyxin, particularly LIM1–2 (7). However, in this case there is no evidence that H-warts/LATS1

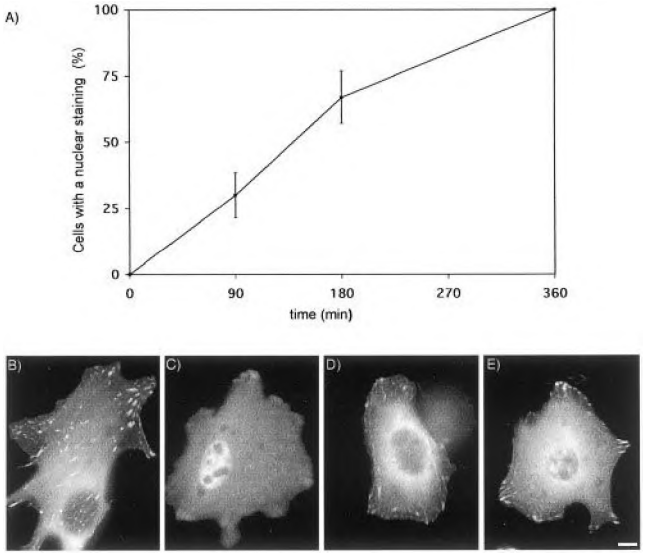

FIG. 8. **Leptomycin B treatment results in nuclear accumulation of GFP-hZyx.** HeLa cells, transiently transfected with GFP-hZyx, were incubated with 20 nM leptomycin B and fixed 0, 90, 180 or 360 min after the addition of the drug. The percentage of cells with a nuclear staining stronger than the cytoplasmic level was calculated at each time point and plotted in *A*. The mean values obtained in two experiments (n = 300 cells) are shown; *error bars* represent the S.D. Fixed cells visualized before (*B*) or after (*C–E*) 90 min of treatment are shown; at this time point, cells with substantial (*C*), minimal (*D*), and moderate (*E*) levels of nuclear zyxin are observed. *Bar*, 10 μm.

resides at focal adhesions; thus, it is not likely that this association is a key determinant of zyxin's ability to associate with focal adhesions. An important goal of future research will be to identify the cellular constituents that play a central role in directing zyxin's LIM domains to focal adhesions.

In addition to the LIM region of zyxin, which displays a robust capacity to localize at focal adhesions, we also detected some residual focal adhesion targeting capacity within the N-terminal proline-rich region of zyxin. This region of zyxin displays docking sites for several proteins including the cytoskeletal proteins, α-actinin, and members of the Ena/VASP family. The results of our analysis illustrate that zyxin targeting is mechanistically complex and that multiple sites on zyxin are likely to contribute to the optimal targeting displayed by the full-length protein.

Our results extend previous studies on zyxin localization (12, 16) and suggest a possible mechanism by which zyxin's subcellular distribution might be regulated. In a 1999 report, Reinhard *et al.* (16) described the expression of a GFP-human zyxin fusion protein lacking the α-actinin binding site (deletion of aa

19–41 from full-length human zyxin) and reported a significant reduction in the ability of the expressed GFP-zyxin to localize to focal adhesions. They interpreted their results to mean that zyxin localizes to focal adhesions by binding α-actinin. We have generated a comparable internal deletion in chicken zyxin and obtained a similar loss of focal adhesion localization. Yet, here we demonstrated that large N-terminal deletions (aa 1–130, 1–244, or 1–305 in chicken zyxin and aa 1–50 or 1–331 in human zyxin), which completely eliminate α-actinin binding sequences, are still capable of focal adhesion targeting. Also in 1999, Drees *et al.* (12) showed that microinjection of a zyxin-derived peptide corresponding to the α-actinin-binding site causes mislocalization of zyxin, suggesting a contribution of this region to focal adhesion targeting. However, as was noted in the original report (12), α-actinin binding is not likely to be sufficient for zyxin targeting, since α-actinin is present at some cellular locations where zyxin fails to accumulate. Drees *et al.* (12) suggested that the presence of the zyxin-derived peptide at focal adhesions might inhibit the accumulation of native zyxin by steric interference. In light of the current results, it is also possible that the peptide interferes with targeting of zyxin by interfering with some aspect of the machinery that regulates focal adhesion targeting of zyxin. Clearly, a simple model in which zyxin localization is a direct consequence of zyxin docking at α-actinin-rich sites is not adequate to explain all of the existing data. The mechanism of zyxin localization must be more complex. It is interesting to consider the possibility that the N-terminal domain of zyxin participates in some regulatory function that modulates the accessibility of targeting sequences in the LIM region. A number of focal adhesion and cytoskeletal proteins including vinculin, Src, and ezrin (2, 46–48) exhibit intramolecular interactions that regulate their binding capacities. In the case of zyxin, a recent report suggests that the availability of the LIM domains for binding to protein partners may indeed be conformationally regulated (7). Perhaps binding of α-actinin (or another partner) to zyxin contributes to changes in zyxin's conformation that can affect focal adhesion targeting and protein docking functions of the LIM domains. In future work, it will be very interesting to define the mechanism by which targeting of zyxin to focal adhesions might be regulated. Our studies clearly show that the multiple regions of the zyxin protein contribute to its capacity to localize at focal adhesions. It is possible that the proline region of zyxin cooperates with the LIM region of zyxin to facilitate optimal targeting to the focal adhesions. Bipartite focal adhesion targeting domains have been reported in several proteins including talin, vinculin, Hic-5, and Ena/VASP (49–52).

Localization of Zyxin to Lamellipodia—The leading edge of a migrating cell represents a region of rapid actin polymerization designed to extend the plasma membrane to both sample the extracellular environment and drive cellular movement (53). At its base, a lamellipodial extension is anchored to the substratum via a collection of dynamic sites called focal complexes (54). As has been reported previously using immunocytochemical methods (12, 16), we find that GFP- and epitope-tagged full-length zyxin is present in lamellipodial extensions. Zyxin is found at levels above cytoplasmic background throughout the lamellipodial extension; however, it should be noted that the lamellipodial accumulation of zyxin is not coincident with the distal lamellipodial concentration of Ena/VASP family members (38). Here we report that sequences in the proline-rich N terminus of zyxin accumulate at the leading edge. Although many of the same proteins are present in the leading edge and the focal adhesions, the demonstration that different regions of zyxin are primarily responsible for its ability to accumulate at different places in cells points out that these sites must have heterogeneous composition or organization. The functional significance of the ability of the proline-rich N terminus of zyxin to accumulate in lamellipodia is not clear.

Nuclear Localization of Zyxin—Zyxin is not detected in cell nuclei at steady state; however, the protein shuttles between the nuclear and cytoplasmic compartments of fibroblast cells (4). Efforts to induce nuclear accumulation of zyxin by the manipulation of tissue culture conditions (*e.g.* cell density), inhibition/activation of various protein kinase cascades, induction of cellular damage, or modification of the cytoskeleton have been unsuccessful (data not shown). However, here we show that one strategy for inducing nuclear accumulation of zyxin is to inactivate its nuclear export signal by deletion or by treating cells with the generalized nuclear export inhibitor leptomycin B. Interestingly, Zeile and colleagues have also reported nuclear accumulation of endogenous zyxin after infection of cells with a vaccinia virus (55). Whether this altered distribution results from specific inactivation of zyxin's nuclear export signal or enhancement of zyxin's import activity remains to be resolved.

The hydrodynamic properties of chicken zyxin indicate that the protein behaves as an elongate monomer of 69 kDa (3), which is too large to diffuse passively through the nuclear pore complex (56). However, zyxin does not contain a consensus nuclear localization signal; thus, it is possible that nuclear localization signal-containing zyxin-binding partners transport zyxin into cell nuclei. Alternatively, zyxin may use an unconventional nuclear import mechanism similar to that described for the cell adhesion and cell signaling protein β-catenin that directly interacts with the nuclear pore complex (57). Our results suggest that both the proline-rich region and the LIM domains of zyxin may contribute to nuclear import. Neither domain is basic, thus accumulation within cell nuclei is likely to occur via a specific mechanism.

Zyxin Plays a Significant Role in the Targeting of Ena/VASP Proteins to Focal Adhesions—The fact that the LIM region of zyxin was effectively incorporated at the focal adhesion, to the exclusion of full-length endogenous zyxin, allowed us to explore the consequences of compromising zyxin function on focal adhesion composition. We previously postulated that zyxin might play an important role in the localization of Ena/VASP proteins at focal adhesions (38), since it displays several docking sites for members of the Ena/VASP family. We show here that, consistent with this possibility, when the focal adhesions are populated with a zyxin species that lacks the proline-rich Ena/VASP docking sites, Ena/VASP family members are substantially mislocalized. We previously observed a reduction in levels of Ena/VASP proteins in focal adhesions when zyxin was misplaced from focal adhesions using an inhibitory peptide (12). Rottner and colleagues also noted a reduction in Ena/VASP proteins at focal adhesions when zyxin is depleted from these sites in response to inhibition of the Rho-kinase (38), although these authors emphasize the conclusion that zyxin is not the sole contributor to Ena/VASP targeting. The results from all investigators appear to be very similar; the elimination of zyxin from focal adhesions clearly diminishes the capacity of Ena/VASP proteins to accumulate there, while not necessarily eliminating it completely. Other constituents of the focal adhesion, such as vinculin, are not typically perturbed under these circumstances, illustrating the specificity of the loss of Ena/VASP family members when zyxin is misplaced.

Although our results point out the importance of zyxin for the targeting of Ena/VASP proteins to focal adhesions, it is important to emphasize that not all targeting of Ena/VASP proteins involves the zyxin family. For example, a zyxin-

independent mechanism is clearly involved in the targeting of Ena/VASP proteins to the distal tips of leading lamellipodia (38) and to sites of T-cell receptor clustering (24). Thus, it appears that multiple distinct mechanisms are involved in the appropriate targeting of Ena/VASP proteins to discrete subcellular domains, with zyxin playing a prominent role at focal adhesions.

Dominant Negative Interference with Zyxin Function Disturbs Cell Morphology and Cytoskeletal Integrity—The mislocalization of full-length zyxin, induced by expression of the LIM region of zyxin, is associated with changes in cell morphology. With high level expression of the LIM region, we observe a retraction of cell borders. This is consistent with our previous results in which we noted the dependence of cell spreading on normal zyxin distribution and function (12, 24). Interestingly, loss of zyxin from focal adhesions has been reported to be an early indicator of focal adhesion disassembly (38). We also observe a thinning of the actin-rich stress fibers, and often, they appear fragmented. These changes do not occur when other sequences are overexpressed in these cells (data not shown). The alterations in the integrity of the actin cytoskeleton observed when full-length zyxin is displaced from focal adhesions could be explained by the loss of some machinery that contributes to actin assembly necessary for stress fiber maintenance. Since mislocalization of Ena/VASP proteins by introduction of (D/E)FPPPP repeats (23, 37) does not disturb the actin architecture of well spread cells, the loss of zyxin partners other than Ena/VASP proteins is probably responsible for the cytoskeletal effects we observe using the dominant negative zyxin construct.

In conclusion, using several methods and two species, we have identified conserved features of zyxin that contribute to its localization to focal adhesions, lamellipodia, and the nucleus. We provide evidence that zyxin plays an important role in the localization of Ena/VASP family members to focal adhesions. In addition, we show that dominant negative interference with zyxin function affects cytoskeletal architecture and cell morphology.

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